Alteration of Topoisomerase II Action Is a Possible Molecular Mechanism of HL-60 Cell Differentiation

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The inhibition of differentiation and persistence of proliferation in cell transformation is probably not only caused by the mutation of single genes. An additional mechanism of transcriptional control, not only of single genes but of gene programs, is possibly the alteration of the topoisomerases. These enzymes regulate the conformation of DNA by twisting and unwinding the double strands. As has been shown previously, only the genes located in relaxed DNA areas are transcribed and, therefore, the topoisomerases can be described as a gene regulation device. We present the hypothesis that topoisomerase II action is not only altered in, but also necessary for, HL-60 granulocytic cell differentiation. Thus, alteration of topoisomerases may well be a molecular mechanism of cellular differentiation.

Introduction

Hematopoiesis is one of the best understood differentiation systems. The knowledge of the differentiation state of the original cell of a leukemic clone or a lymphoma allows the exact diagnosis and choice of the proper therapy. This is the basis for clinical diagnostic systems such as the FAB-classification (French-American-British) of leukemias and the Kiel classification of malignant lymphomas. The Kiel classification enables one to define the malignant cell as a cell that is disturbed and blocked in its differentiation. However, a therapy derived from the biology (e.g., differentiation induction) has not been established. One reason is the lack of knowledge of the molecular mechanisms of cell differentiation and transformation.

To date only a very small minority of human malignomas could be identified as being caused by chromosomal translocations or point mutations in single genes. For example, less than 30% of human malignomas have a point mutation in the *ras* gene family. These facts suggest the existence of additional mechanisms that could very likely involve the transcription regulation not only of single genes but of whole gene programs.

It has been discussed previously that a change in DNA cytosine methylation is one regulatory mechanism in the long-term control of gene transcription. This obviously does not play a role in the granulocytic differentiation of HL-60 cells, as we have reported in a recent publication (*1*).

Another possible mechanism is a change in the DNA conformation. There are two typical DNA conformations: supercoiled and relaxed. The enzymes that regulate conformational changes of the DNA are the topoisomerases. It is a well-known fact that only genes that are located in relaxed areas of the DNA are transcribed (*2*). Thus, the topoisomerases are unique gene transcription regulating enzymes.

Two different types of topoisomerases are described and can be distinguished by their different assay requirements: the topoisomerase type II depends on Mg^{2+} and ATP, whereas the topoisomerase type I is ATP independent (*3*). Both enzymes have as well DNA-twisting as DNA-unwinding activities. The factors that influence the direction are not completely understood, but by varying the assay conditions the topoisomerase-action can be separated in several different steps (*4*).

In this paper we present evidence that a change in topoisomerase II activity is one additional mechanism in the regulation of genes that control hematopoetic cell differentiation. In the experiments shown, we used the HL-60 cell system and induced granulocytic differentiation with the help of dimethylsulfoxide (DMSO).

Materials and Methods

Chemicals, Media, and Cell Culture

The chemicals and media used are commercially available: RPMI 1640 medium, fetal calf serum, HEPES (Bethesda Research Laboratories, Karlsruhe, FRG), EDTA, DMSO, and mercaptoethanol (Sigma Chemical Co. Ltd., Deisenhofen, FRG).

HL-60 cell culture (from American Type Culture Col-

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lection, Rockville, MD) as well as differentiation induction were performed as described previously (1).

Assessment of Topoisomerase II Activity

Nuclei of 3×10^8 cells were isolated as follows (all steps on ice): wash twice with isotonic buffer (e.g., PBS), resolve in 8 mL lysis buffer (0.3 M sucrose, 0.5 mM EGTA, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.05 mM spermidine, 15 mM HEPES, pH 7.5, 14 mM 2-mercaptoethanol), add 40 μL prewarmed Triton X-100, gently mix for 15 min on ice, sediment (1000g, 5 min, 0°C in a swing-out rotor), count nuclei after staining with trypan blue, resuspend in 2 mL lysis buffer (without Triton X-100), sediment through 7 mL 30% sucrose in lysis buffer. Recovery should be almost 100%. Nuclei can be stored at −70°C for months in 100-μL aliquots, 2×10^5 nuclei per microliter storage buffer [20 mM tris, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM phenylmethylsulfonyl fluoride (PMSF) stock 200 mM in isopropanol or 10^{-4} M trasylol, 50% glycerine].

Topoisomerase II solution was made as described with slight variations (5): nuclei were collected by centrifugation (200g for 10 min) and resuspended at 3×10^7 mL in 5 mM potassium phosphate, pH 7.5, 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride. NaCl (5 M) was added to a final concentration of 0.35 M, and the nuclei were mixed gently on a Vortex mixer, incubated on ice for 60 min, and sedimented (1000g for 10 min).

For relaxation experiments a 5-μL aliquot of the supernatant fraction was mixed with 5 μL reaction buffer (100 mM Tris-HCl, pH 7.5, 200 mM KCl, 1 mM EDTA, 0.3 μg bovine serum albumin) and 200 ng pBR322 plasmid DNA. For topoisomerase II activation, 10 mM MgCl₂ and 10 mM ATP was added fresh. After incubation at 37°C for 60 min, the DNA was electrophoresed in a 1% agarose gel and visualized by staining with ethidium bromide after electrophoresis.

We used a large-scale plasmid preparation and isolation by the alkaline method described by Maniatis (6) to isolate PBR322 plasmids from E. coli.

Results

A marked increase of topoisomerase II activity can be monitored in granulocytic HL-60 cell differentiation, as shown in Figure 1. The HL-60 cells had been induced to differentiate by culturing for 5 days in the presence of DMSO (1% v/v). Differentiation was confirmed with the help of the following markers: cell growth, viability, and nitro-blue tetrazolium-reduction (NBT test). The differentiated cells accumulated in the G_0 phase and stopped dividing. Viability was always more than 80%.

To distinguish between topoisomerase I and topoisomerase II activity, we performed experiments of the kind shown in Figure 2. It has been described previously that topoisomerase II is activated and topoisomerase I
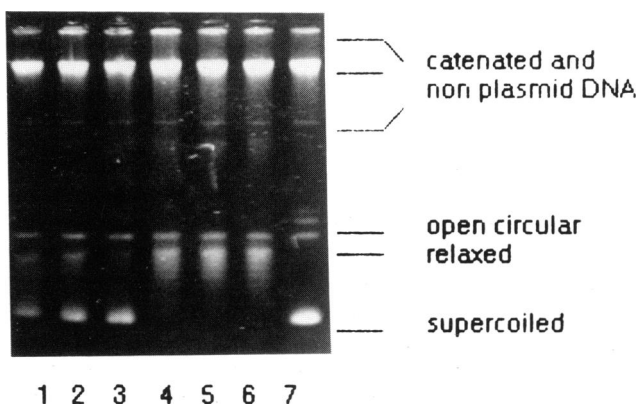

FIGURE 1. Topoisomerase II activity of undifferentiated and granulocytic differentiated HL-60 cells. Approximately 200 ng plasmid DNA and nuclear extract from 10^5 cells were used per experiment. Lanes 1–3, HL-60 cells (1:2, 1:4, 1:8); lanes 4–6, HL-60 cells/DMSO (1:2, 1:4, 1:8); lane 7, control (pBR322, 200 ng).
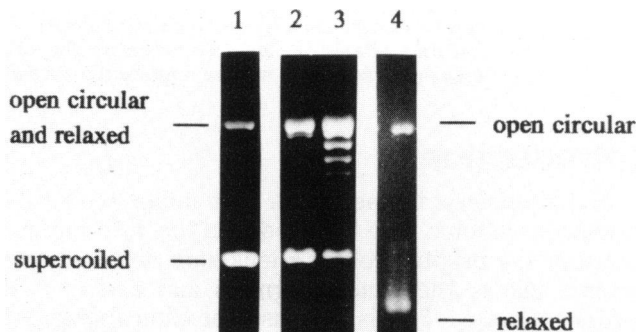

FIGURE 2. Differentiation between topoisomerase I and topoisomerase II activity and the open circular and relaxed form of pBR322 plasmid. Approximately 250 ng plasmid DNA and nuclear extract from 10^5 cells were used per experiment. Lane 1, pBR322, 250 ng; lane 2, pBR322 plus extract; lane 3, pBR322 plus ATP and Mg; lane 4, pBR322, extract, ATP, Mg, and EtBr.

is blocked by the addition of ATP to the reaction buffer (7). We found very little relaxation activity without the addition of ATP, which we contribute either to a slight topoisomerase I activity or to the endogenous ATP content in the nucleic extracts we used (~0.1 mM). From these experiments we conclude that the relaxation activity we found is mainly due to topoisomerase II activity and not to topoisomerase I.

By intercalation of ethidium bromide, the open circular plasmid DNA and the supercoiled plasmid DNA can be separated. The open circular form results from single strand nicks that occur on isolation of the plasmids from E. coli bacteria. Addition of ethidium bromide to the gel running buffer results in intercalation into the DNA molecule, which increases the spacing of successive base pairs and subsequently decreases the

pitch of the helix. The two forms can then be separated by their different migration speeds in agarose gel electrophoresis as shown in Figure 2.

Discussion

Topoisomerase II inhibitors are strongly cytostatic substances because topoisomerase action is needed for cell division as well as for gene transcription. Addition of these inhibitors in sublethal concentrations to the medium of HL-60 cells can also induce differentiation and block the induction of differentiation by DMSO. We have published both observations previously: granulocytic differentiation can be induced by the addition of mitoxantrone 2×10^{-8} M (8), and DMSO-induced differentiation can be inhibited by the addition of 4'-(9-acridinylamino)methanesulfon-M-aniside (m-AMSA) 10^{-8} M (9). These results, which appear contradictory at first, represent the highly specific mode of action of topoisomerase II. In order to control gene transcription the enzyme has to recognize specific DNA-binding sites; the existence of these sites had been shown several times. Moreover, topoisomerase II induces cleavage sites in promoter-active DNA regions, which has been described previously for the c-*myc* proto-oncogene (10) and the c-*fos* proto-oncogene (11). Both genes obviously play important roles in proliferation and differentiation of hematopoetic cells.

In this paper we have shown that topoisomerase II activity increases in granulocytic differentiated HL-60 cells (Fig. 1), in spite of the markedly reduced proliferation capacity of the differentiated cells. We attribute this result to the higher transcriptional rates of the specific genes that code for the specific abilities of the differentiated cells (e.g., reductase activity, phagocytosis). Combination with the previously published results described above shows that topoisomerase II action is not only altered in but is also necessary for granulocytic HL-60 cell differentiation.

In the experiments described here, we used assay conditions that prefer topoisomerase II activity. We confined ourselves to topoisomerase II activity because it had been shown that this enzyme is the molecular target for a number of clinically relevant cytostatics, such as anthracyclines (e.g., doxorubicin, mitoxantrone), podophyllotoxins (e.g., etoposid, tenoposid), and synthetic substances (e.g., m-AMSA) (12). These substances act via inhibition of an intermediate DNA-enzyme complex, which results in DNA double-strand breaks with covalently bound proteins (the topoisomerase subunits). The number of these topoisomerase II-induced DNA double-strand breaks can be correlated with the cytotoxicity of the substances (13).

This way of cytostatic action can also be used to define the DNA-binding sequences of topoisomerase II by using restriction enzyme fragment length polymorphism (RFLP analysis) of the DNA areas of interest to determine the sites of the strand breaks. If an altered topoisomerase II was one of the molecular mechanisms of HL-60 cell differentiation, one would expect not only an altered topoisomerase activity but also altered DNA binding sites in order to relax different DNA regions. We plan to test this hypothesis in the near future.

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